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(54) Title: APOLIPOPROTEIN E2 AND TREATMENT OF ALZHEIMER'S DISEASE

(57) Abstract

Recombinant apolipoprotein E2 (apoE2) which binds to amyloid peptide Aβ.

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APOLIPOPROTEIN E2 AND TREATMENT OF ALZHEIMER'S DISEASE

Field of the Invention

This invention relates to recombinant DNA, protein purification, and Alzheimer's disease.

Background of the Invention

Alzheimer's disease has two major neuropathological characteristics. The first is the presence of intraneuronal neurofibrillary tangles, the major protein component of which is hyperphosphorylated tau, a microtubule-associated protein. Electron microscopy shows neurofibrillary tangles to comprise paired helical filaments ("PHF") (Selkoe (1991) Neuron 6:487-498).

The second major characteristic is the presence of extracellular amyloid deposits in the brain. Amyloid deposits consist of polymers of the amyloid peptide Aβ, which is proteolytically derived from amyloid precursor protein ("APP").

Apolipoprotein E ("apoE") is a 34.2 kDa protein synthesized by the liver and various peripheral tissues, including kidney, adrenal gland, astrocytes and reticuloendothelial cells. ApoE is synthesized as precursor ("preapoE") with an 18-amino acid signal peptide. After the intracellular cleavage of the signal peptide, apoE is glycosylated with carbohydrate chains containing sialic acid and secreted as sialo apoE. It is subsequently desialated in plasma. For a discussion of apoE biosynthesis processing and other properties, see: Zannis et al. (1993) Adv. Hum. Genet. 21:145-319; Zannis et al. (1984) J. Biol. Chem. 259:5495-5499; and Zannis et al. (1986) J. Biol. Chem. 261:13415-13421.

Following synthesis, apoE is incorporated into lipoproteins and directs their catabolism, following recognition and binding by cell surface receptors. ApoE can be isolated from plasma and culture media of HepG-2 cells as a component of lipoprotein particles.

There are three common alleles that encode apoE in humans. The three alleles designated $\epsilon 4$, $\epsilon 3$, and $\epsilon 2$ give rise to three homozygous phenotypes (i.e., E4/E4, E3/E3, and E2/E2) and three heterozygous phenotypes (i.e., E4/E3, E3/E2, and E4/E2)(Zannis and Breslow (1981) Biochemistry 20:1033-1041; Zannis et al. (1981) Am. J. Hum. Genet. 33:11-24). The three different human apoE isoproteins, apoE4, apoE3, and apoE2, result from mutations at amino residues 112 and 158. ApoE4 contains Arg at position 112 and Arg at position 158. ApoE3 contains Cys at position 112, and Arg at position 158. ApoE2 contains Cys at positions 112 and 158.

Mutations in apoE which affect its recognition by the LDL receptor are associated with type III hyperlipoproteinemia and premature atherosclerosis. See, e.g., Goldstein et al. (1983) in: The Metabolic Basis of Inherited Disease (Stanbury et al., eds.), McGraw-Hill, New York, pp. 672-712. The apoE mutation which results in the apoE4 phenotype occurs with increased frequency in patients with late onset of Alzheimer's disease (Corder et al. (1993) Science 262:921-923).

ApoE and Alzheimer's Disease

ApoE synthesis is increased following injury and is implicated in the growth and repair of the nervous system during development or after injury (Boyles et al. (1989) J. Clin. Invest. 83:1015-1031). In the peripheral nervous system, the synthesis of apoE increases 250-350 fold within 3 weeks of sciatic nerve crush, and apoE constitutes 5% of the total soluble extracellular

protein. Recently, it has been shown that apoE is found in the brain lesions of AD patients and that the apoE4 phenotype predominates in patients with late onset familial AD (Corder et al. (1993) Science 262:921-923). It has been suggested that apoE is a risk factor and contributes to the pathogenesis of the disease. Two hypotheses have been advanced to explain the involvement of apoE in AD. The first hypothesis implies extracellular interaction of apoE with the 42 amino acid long amyloid peptide β (Aβ). Initial observations indicated that apoE from cerebrospinal fluid binds with high affinity to Aβ (Wisniewski et al. (1993) Biochem. Biophys. Res. Commun. 192:359-365; Strittmatter et al. (1993) Proc. Natl. Acad. Sci. USA 90:1977-1981). *In vitro* experiments have shown that apoE can form a complex with Aβ peptide which is stable following boiling for 5 min at 2% SDS. Formation of stable complexes of apoE4 with Aβ is fast, compared to formation of complexes of apoE3 with Aβ.

Electron microscopy studies showed that a combination of apoE4 with Aβ resulted in the formation of monofibrils 7 nm in diameter. The matrix of monofibrils formed with apoE4 was much denser than that with apoE3 (Sanan et al. (1994) J. Clin. Invest. 94:860-869).

A second hypothesis is that apoE3 binds to tau, and protects it from phosphorylation in the microtubule-binding domain, while apoE4 does not bind to tau (Strittmatter et al. (1994) Exper. Neurol. 125:163-171). As a result, tau gets hyperphosphorylated and can no longer bind efficiently and stabilize microtubules, leading to the formation of neurofibrillary tangles inside neurons.

Summary of the Invention

We have discovered that glycosylated apolipoprotein E2 having a conformation which is the same as, or which closely approximates, its native

conformation, i.e., it is not denatured and then renatured, and exists in lipid-free as well as lipid-bound forms, binds to synthetic peptide amyloid peptide Aβ with a higher affinity than do apolipoproteins E3 and E4.

Accordingly, the invention features an isolated apolipoprotein E2 capable of specifically binding to amyloid peptide Aβ with a higher affinity than apolipoproteins E3 or E4; the apolipoprotein E2 can be produced by (a) transforming a eukaryotic (preferably mammalian) host cell with DNA encoding apolipoprotein E2; (b) culturing the host cell; and (c) isolating the apolipoprotein E2 under non-denaturing conditions. As used herein, "apolipoprotein E2" refers both to the naturally-occurring amino sequence of the protein, and to variants in which there are one or more amino acid substitutions which do not substantially alter the conformation or other properties of the molecule. Examples of such mutant forms of E2 are given below in Table 1; these mutants not only retain native conformation when expressed in mammalian host cells, they exhibit superior binding properties compared to the native sequence.

Apolipoprotein E2 can be isolated under nondenaturing conditions by subjecting the growth medium from a culture of mammalian cells that express recombinant apolipoprotein E2 to cation exchange chromatography to produce an apolipoprotein E2-enriched cation exchange protein fraction, and then subjecting the resulting pool of apolipoprotein E2-enriched cation exchange fractions to anion exchange chromatography, thereby producing an anion exchange fraction highly enriched for apolipoprotein E2.

The invention also features a method for inhibiting the progression of Alzheimer's disease in a

patient with early onset Alzheimer's disease, or inhibiting the onset of Alzheimer's disease in a patient genetically at risk of developing Alzheimer's disease, by administering to the brain of the patient an effective amount of apolipoprotein E2 which is glycosylated, exists in lipid-free or lipid-bound forms, and has native or close to the native conformation produced by brain cells.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Drawings

Fig. 1 is a schematic representation of the steps leading to production of plasmids of the invention.

Fig. 2 is a series of gel electrophoresis and autoradiography panels of apoE2 secretion from cells of the invention.

Fig. 3 is a series of panels illustrating apoE2, E3, and E4 expression by various cell types using the Semliki Forest Virus expression system.

Fig. 4 is a pair of panels illustrating apoE expression in a bioreactor setting.

Fig. 5 is a set of panels showing elution profiles of apoE from ion exchange columns.

Fig. 6 is a set of panels showing binding to Aβ of apolipoprotein E obtained by expression in eukaryotic cells. The apoE experiment shows that the ability of apolipoprotein E produced by eukaryotic cells to bind to Aβ follows the order: apolipoprotein E2 > apolipoprotein E3 > apolipoprotein E4.

Fig. 7 is a set of panels showing binding to Aβ of apolipoprotein E3 and apolipoprotein E4 obtained by the baculovirus expression system. The ability of apoE3 and apoE4 forms thus obtained to bind to Aβ is different from that of apoE expressed by eukaryotic cells (Fig. 6).

Fig. 8 is a set of panels showing binding to Aβ of VLDL, apoE3 and apoE4. The ability of apoE3 and apoE4 forms thus obtained to bind to Aβ is different from that of apoE expressed by eukaryotic cells (Fig. 6).

Fig. 9 is a panel showing distribution of apolipoprotein E secreted by C127 cells to lipid-free and lipid-bound forms.

Fig. 10 is a diagram predicting binding of apolipoprotein E2 derivatives obtained from eukaryotic cells to Aβ which differ from the binding of naturally occurring apolipoproteins E3, E4 and native-sequence E2.

Detailed Description

The present invention provides recombinant apolipoprotein E2 in its native conformation. Because of its native conformation, the apoE2 of the invention binds to the Aβ peptide with greater affinity than apoE3 or apoE4.

Generation of cell lines of C127 cells expressing apoE2, apoE3 and apoE4.

We have generated a recombinant C127 cell line that secretes apoE2. This cell line has been generated as follows: The apoE cDNA we isolated previously (Zannis et al. (1984) J. Biol. Chem. 259:5495), amplified and mutagenized by PCR, was excised, with nucleases AatII and DraII, from the original plasmid, and subcloned into the SmaI site of the pUC19 plasmid to produce the pUC-E3 derivative. This was followed by excision of the apoE DNA with BamHI and BglII. The pUC-E3 plasmid was amplified and mutagenized by PCR as described in Fig. 1A, using a set of 5′ and 3′ amplification primers and a set of mutagenic primers. The mutagenic primers were designed to replace Cys-112 with Arg, and to replace Arg-158 with Cys.

The 5′ external amplification primer extended from nucleotides 223 to 241 of the apoE gene (sequence numbering of Karathanassis, Haddad, Salmon and Zannis, Biochemistry and Biology of Plasma Lipoproteins 1985, pp. 475-493). The 3′ external amplification primer extended between nucleotides 1102-1082 in the 3′ region of the cDNA. One set of mutagenic sense and antisense oligonucleotides covered codons 154-161 (nucleotides 576-595) for alteration of Arg-158 to Cys-158 in order to produce apoE2. Another set of mutagenic sense and antisense oligonucleotides covered codons 108-113 (nucleotides 438-457) for the change of Cys-112 to Arg-112 in order to produce apoE4.

For alteration of Arg-158→Cys, two separate amplifications were utilized. The first utilized the 5′ external primer and the antisense mutagenic primer covering codon 158. The second utilized the 3′ external primer and the sense mutagenic primer covering codon 158. An aliquot of 4% from each reaction was mixed and amplified with the 5′ and 3′ external primers. The amplified fragment containing the Arg-158→Cys mutation was used to replace the corresponding region in the pUC-E3 plasmid, as shown in Fig. 1A, giving rise to pUC-E2 plasmid. Similarly, the amplified fragment containing the Cys-112→Arg mutation was used to replace the corresponding region of the pUC-E3 plasmid, as shown in Fig. 1A, giving rise to pUC-E4 plasmid (Fig. 1A).

The DNA insert obtained from the pUC-E2 derivative was initially cloned into the BamHI site of the pcDNA under the control of the CMV pcDNA promoter or into the BamHI and BglII site of Semliki Forest Virus vector (pSFV) to generate the pcDNA-E2 derivative and the pSFV-E2 derivatives. The apoE2 insert was then excised from the pUC-E2 derivative, blunt-ended with the Klenow

fragment of the DNA polymerase I, and ligated to XhoI linkers digested with XhoI and cloned into the XhoI site of pBMT3X vector to generate pBMT3X-E2, expression plasmid (Fig. 1B). A similar approach was utilized to generate the pBMT3X-E3 and E4 plasmids.

The pBMT3X is a bovine papilloma virus-based vector in which the gene of interest is transcribed under the control of the mouse metallothionein I promoter. The vector also contains the human metallothionein I gene, which confers resistance to 10 μM $CdCl_2$ (Cladaras et al. (1987) J. Biol. Chem. 262:2310-2315).

To generate stable cell lines expressing apoE2, cells were transfected with the pBMT3X-apoE2 expression plasmid by the Calcium Phosphate DNA co-precipitation method (Graham and van der Eb (1973) Virology 52:456). Permanent cell lines were selected for their resistance to 10μM $CdCl_2$. Resistant colonies were isolated with cloning cylinders and grown to 90% confluency.

To characterize the protein secreted by this cell line, the cell line was labelled with ^{35}S-methionine for 2 hours. Media and cell lysates were immunoprecipitated with rabbit anti-apoE, mixed with 15 μg HDL containing apoE3, and analyzed with two-dimensional gel electrophoresis (PAGE) and autoradiography. In this analysis, proteins are separated on the basis of their charge by isoelectric focusing and on the basis of their size by SDS-PAGE.

The gel obtained from this analysis showed the position of the marker plasma apoE3 (Fig. 2A,B,C), and the autoradiogram showed the position of the newly synthesized apoE2 protein (Fig. 2A',B',C'). Superimposition of the gel on the autoradiogram established the charge and size differences between the plasma apoE3 and the newly synthesized protein (Fig. 2A",B",C"). This analysis, shown in Figs. 2A-C",

established that the newly synthesized apoE2 is more acidic by one and two positive charges than apoE3 and apoE4, respectively. The analysis also shows that the secreted protein is extensively modified and resembles the protein synthesized by hepatic and other types of cells (Zannis et al. (1986) J. Biol. Chem. 261:13415-13421).

We have also expressed the apoE2 in Cos-1 cells using the pcDNA vectors under the control of the CMV promoter.

Expression of ApoE cDNA Using the Semliki Forest Virus Expression System

In addition to apoE2 expression in C127 cells, described above, we have also used other eukaryotic expression systems for expression of apoE2, apoE3 and apoE4 based on Semliki Forest Virus (SFV) replicon. These systems consist of two plasmids - pSFV1 and pSFV-helper-2. The first plasmid represents the cDNA copy of Semliki Forest Virus genome with a deleted subgenomic (26S) coding region for the structural proteins of the virus, followed by a cassette of polylinker (BamHI-XmaI-SmaI) and translational stop codons in all three reading frames. The cDNA of human apoE2 was cut with BglII and BamHI, present in the polylinker region of pSFV, and inserted via a BamHI BglII site to create vector pSFV apoE2. In this vector, apoE2 cDNA expression is driven by the 26S promoter in the context of SFV genome. The second plasmid is pSFV-helper 2, which retains the 5′ and 3′ signals needed for RNA replication, as well as the complete structural region, including its promoter, but which lacks a region required for packaging of SFV RNA into nucleocapsids.

The pivotal step in the development of the pSFV expression system is helper catalyzed packaging of

pSFVcE2 RNA. The system allows rapid transient expression of apoE2 cDNA mutants, allowing fast testing of their functions, physicochemical properties, and neuronal toxicity.

Initially, plasmid pSFV apoE2, or pSFV-helper 2 was transcribed *in vitro* under the control of an SP6 promoter. Both SFV apoE2 and SFV-helper 2 RNAs were then cotransfected by electroporation into BHK cells. In the electroporated cells, the SFV apoE2 RNA encodes the enzymes for RNA replication (amplification) and transcription, whereas SFV helper 2 RNA supports the production of the structural proteins via its subgenomic region. Since the helper lacks the region required for RNA packaging, RNA derived from this vector will not be packaged. Thus, transfections with recombinant and helper RNA produce only virus particles that carry recombinant RNA.

The viruses produced provide a one-round virus stock. After infection of cells with the recombinant virus, the cells will only express the heterologous protein (apoE2). This virus stock was utilized to infect BHK cells or primary astrocytes obtained from mice deficient in apoE. For this analysis, confluent BHK cells or apoE deficient mouse primary astrocyte cultures were infected with recombinant SFV apoE2, virus at multiplicity of infection of approximately three infective units per cell for 12 hrs and grown in serum-free BHK medium for 24 h. The viral titer was estimated between 10^7 and 10^8 infectious units/ml. Aliquots of 50 μl medium were removed and analyzed by SDS-PAGE and immunoblotting (Fig. 3). The above description demonstrates that recombinant, glycosylated apoE2 can be efficiently produced in a variety of eukaryotic cells. The level of expression estimated from the intensity of the stained bands is approximately 5-10 μg apoE/10^6 cells/1 ml/24 hrs.

Apolipoprotein E4 and Apolipoprotein E3 Were Expressed in a Similar Way Using the SFV Expression System.

Large-Scale Growth of ApoE2 Producing Cells in Bioreactors (Verax System 10)

The Verax bioreactors consist of a vertical column containing microspheres in suspension (Fig. 4A). The microspheres are made of collagen and are hollow with a pore diameter which allows cell attachment and growth. Cell densities of 10^8 cells/ml are achieved. The microspheres are kept in suspension by the flow of incoming culture medium. A gas exchange unit provides the required O_2 and removes excess CO_2. Temperature control keeps the medium at 37°C. The medium is circulated by a pump through the bioreactor and the gas exchange unit. Using the Verax System-10 Bioreactors the culture medium contain 30-50 μg apoE/ml (Fig. 4B).

The medium of C127 cells expressing apoE2, apoE3 or apoE4 was purified by a scheme employing a dextran sulfate sepharose column and DEAE sepharose column fractionation.

Briefly, a 20 ml dextran sulfate-sepharose column was equilibrated with 20 mM Tris-HCl, 0.2 M NaCl, pH 7.4. A total of 1 liter of apoE-containing culture medium was adjusted to 0.2 M NaCl and loaded on the column at a flow rate of ~80 ml/hr. The column was washed with 200 ml of 20 mM Tris-HCl, 0.2 M NaCl, pH 7.4 at the same flow rate. The column was eluted with a 120 ml 0.2-1.0 M NaCl gradient in 20 mM Tris-HCl, pH 7.4 at the same flow rate; fractions of 2 ml were collected. The apoE-containing samples eluted from the dextran sulfate sepharose column were adjusted to 50 mM NaCl by dilution with 20 mM Tris-HCl pH 7.4 and were loaded on a 10 ml DEAE column. The column was eluted with 70 ml of 0.05-0.75 M NaCl gradient in 20 mM Tris-HCl pH 7.4.

Properties of Apolipoproteins E3, E4 and E2 Produced by Mammalian Cells and Other Sources.

Binding of ApoE2, ApoE3 and ApoE4 Produced by Cell Cultures to the Synthetic Amyloid Peptide Aβ.

For this analysis, 1 μg of apoE2 or apoE3 or apoE4 was diluted with 9 μl H_2O containing either 0 or 4 μM Aβ (40 amino acid residues in length) in 20 mM tris pH 8.0 buffer and incubated at 25°C for 12 hrs. Incubation was terminated by adding equal volume 2X Laemmli buffer (4% SDS without β-mercaptoethanol) and boiling for 5 min. The mixture was analyzed by gel electrophoresis in two gels (10% polyacrylamide - 2% SDS) and Western blotting. After blotting one of the filters (Panel A) was treated with mouse monoclonal antibody against Aβ (Fig. 6A) and the other two panels (Panels B&C) with goat polyclonal antibody against apoE.

This experiment shows that recombinant apoE2, apoE3 and apoE4 form stable diffused complexes with Aβ in solution (Fig. 6A). Each such complex is stable following boiling in 2% SDS. The diffused nature of the complex may result from the fact that apoE produced by these cell lines is highly modified with carbohydrate chains containing sialic acid. There is no formation of complexes with higher Mr with any of the isoforms. The formation of complexes can be seen also in the first three lanes of Fig. 6B in lane 4 which do not contain Aβ. In this experiment equal quantities of apoE (1 μg) and Aβ (4 μg) were utilized for all the isoforms. The analyses of Fig. 6A-C show that the ability of apoE produced by these cells to bind to Aβ follows the order apoE2>apoE3>apoE4. Similar results are obtained with apoE produced by apoE produced by the different expression systems described before. This binding behavior of apoE is different from that of apoE produced by the baculovirus expression system (Fig. 7) or VLDL (Fig. 8)

(Strittmatter et al. (1993) Proc. Natl. Acad. Sci. USA 90:8098-8102).

The experiments of Fig. 6A-C show that recombinant apoE2, apoE3 and apoE4 form stable complexes with Aβ. These complexes are visible in the first three lanes of Fig. 6A,B, which contain Aβ but not in the corresponding lanes of Fig. 6C, which does not contain Aβ. This analysis establishes unequivocally that the binding of newly synthesized apoE secreted into the cell medium which consist mostly of modified forms of apoE with Aβ follows the order apoE2>apoE3>apoE4.

In other experiments we have analyzed the apoE secreted by cell cultures by density gradient ultracentrifugation and immunoblotting. This analysis showed that approximately 45% of apoE is distributed in lipoprotein particles which float in the HDL region in d=1.08-1.20 g/ml, 40% in lipid-poor particles which float in the d=1.22-1.28 g/ml region, and 15% in a lipid-free fraction d>1.33 g/ml (Fig. 9). These different types of particles participate to different degrees in the formation of complexes with Aβ.

According to epidemiological data (Corder et al. (1993) Science 262:921) and the findings of Fig. 6A-C, increased binding of apoE isoforms to Aβ correlates negatively with the risk of developing Alzheimer's disease. Derivatives of apoE2 generated by *in vitro* mutagenesis containing one or more amino acid substitutions which do not substantially alter the conformation or other properties of the molecule may surpass the ability of the naturally occurring apoE2 form to bind Aβ. These derivatives (examples are shown in Table 1) will provide a series of experimental points to assess the binding behavior of apoE (Fig. 10). Taking into account the reported progress in the generation of an animal model of AD (Gomes et al. (1995) Nature

373:523), one approach to further establish the relationship between the binding of apoE to Aβ and AD will be as follows: mice expressing natural or mutant apoE forms in an apoE deficient background (Plum et al. (1992) Cell 71:343-353) will be mated with the AD-prone mouse generated by Athena Neuroscience (Gomes et al. (1995) Nature 373:523). Analysis of the offspring will provide information as to whether a specific apoE variant protects or accelerates the development of the AD phenotype in relationship to the parent AD mouse. For instance, crossing of the AD prone mouse with the EX1 or EX6 mouse will confirm that strong binding of apoE to Aβ is beneficial and weak binding is detrimental. If strong binding is beneficial, we will anticipate that the EX6 form will protect from AD when it is introduced into the AD-prone mouse, whereas EX1 will accelerate the development of AD.

Formulation and Administration

To be effective, the apoE2 of the invention should come in contact with neuronal cells of the brain, or with the physiological fluids in the immediate vicinity of such cells. The apoE2, in that environment, binds to the Aβ-amyloid peptide, or to β-amyloid precursor protein; when it binds to precursor protein; it prevents the precursor from progressing to the next stage, which is cleavage to form the β-amyloid protein characteristic of Alzheimer's disease. When apoE2 binds to Aβ-amyloid peptide, such binding inhibits progression of Alzheimer's disease by preventing formation of the extracellular amyloid plaques.

Generally, there are several approaches to bring apoE2 into contact with brain cells or to remove Aβ from the brain.

It has been shown that noncovalent association of apoE with lecithin phospholipids leads to the formation of discoidal particles (Innerarity et al. (1979) J. Biol. Chem. 254:4186-4190). ApoE also associates with triglyceride-rich phospholipid emulsions (Derksen and Small (1989) Biochemistry 28:900-906).

In one method, apoE2 produced by eukaryotic cells will be administered into the ventricles of the brain and will enter into the cerebrospinal fluid where it will be bound to Aβ.

In another method, apoE2 phospholipid vesicles or triglyceride phospholipid emulsion will be administered into the ventricles of the brain. The Aβ will bind to the vesicles or the emulsions and will be removed from the brain.

In a third method, apoE2 phospholipid vesicles or the triglyceride phospholipid emulsions will be administered intravenously. The complexes will cross the blood/brain barrier by interactions with the apoE receptor present in the blood/brain barrier.

In a fourth method, biotinylated polyamides will be conjugated to streptavidin conjugated apoE2 according to Partridge et al. (1995) Proc. Natl. Acad. Sci. USA 92:5592-5596). Following intravenous administration, the complex will cross the blood/brain barrier by interaction with the apoE receptor present in blood brain barrier.

Free apoE2 or apoE2 associated with lipids can be administered to the ventricle of the brain by means of a stereotaxic instrument which is commercially available. The stereotaxic coordinates of the insertion point on the skull is marked by a sterile pencil. A hole of 2-3 mm is made in the skull. The dura is punctured and is covered with saline soaked gel-foam using the stereotaxic holder, and a thin tube is lowered into the ventricle and is connected with the infusion pump that will deliver the

apoE2 in lipid-free or lipid-bound form into the ventricle.

The apoE2 solution or apoE2 phospholipid vesicles or apoE2 triglyceride phospholipid emulsions are loaded into the intraventricular pump, which has been implanted into the patient by known techniques. Each apoE2 preparation is continuously infused into the ventricle of the patient's brain, at a rate sufficient to deliver 500 to 1000 μg of protein per 75 kg body weight of the patient per day.

Another mode of administration is to inject the protein, dissolved in a carrier liquid, into the spinal column of the patient so that it contacts the patient's cerebrospinal fluid.

Another mode of administration is intravenous injection of apoE2 phospholipid vesicles or apoE2 triglyceride phospholipid emulsions or conjugates of apoE2 with polyamides in physiological solutions containing 2 mg apoE2/ml. Ten mls of apoE2-containing solution will be injected twice per day.

Generally, the apoE2 of the invention can be administered to three classes of patients:

1. Patients who have been diagnosed with Alzheimer's disease, and who are exhibiting moderate to severe symptoms of the disease;

2. Patients who are exhibiting early-stage symptoms of Alzheimer's disease, and who are therefore suspected of having the disease; and

3. Asymptomatic patients who are over the age of 45 and who have a family history of Alzheimer's disease; one class of such patients are those carrying two copies of the apoE4 gene.

An alternative method of treatment is to administer DNA encoding apoE2 rather than apoE2 to protein. This gene therapy method is preferably carried

out using either a retroviral vector or an expression vector containing apoE2-
encoding DNA, suspended in a liquid carrier; the suspension is administered either to the cerebrospinal fluid or the brain ventricle in the same manner that the protein in solution is administered. Suitable plasmid and retroviral vectors are well known and publicly and commercially available.

Other embodiments are within the following claims.

What is claimed is:

Claims

1. Recombinant apolipoprotein E2 (apoE2) which binds to amyloid peptide Aβ or to amyloid β precursor protein.

2. The apoE2 of claim 1 wherein said apoE2 has a higher affinity for Aβ than do apolipoproteins E3 and E4.

3. The apoE2 of claim 1, having one of the amino acid sequences shown in Table 1.

4. The apoE2 of claim 3 wherein said apoE2 has a higher affinity for Aβ than does the native-sequence form of apolipoprotein E2.

5. The apoE2 of claim 1 wherein said apoE2 is glycosylated and is produced by the process of

(a) transforming a eukaryotic host cell with DNA encoding apolipoprotein E2,

(b) culturing said host cell in a growth medium, and

(c) isolating said apolipoprotein E2 under non-denaturing conditions.

6. The apoE2 of claim 3 wherein said eukaryotic host cell is a mammalian cell.

7. The recombinant apolipoprotein E2 of claim 1, wherein Cys 112 of the native molecule is replaced with Arg.

8. A recombinant mammalian cell that expresses recombinant apolipoprotein E2.

9. The cell of claim 8, wherein said apolipoprotein E2 binds to amyloid peptide Aβ with a higher affinity than do apolipoproteins E3 and E4.

10. The cell line of claim 8, wherein said apolipoprotein E2 has a non-native amino acid sequence and binds to amyloid peptide Aβ with higher affinity than does native-sequence apoE2.

11. A transgenic mouse having an apoE-deficient background, wherein said mouse expresses human apoE2 in neurons of its brain.

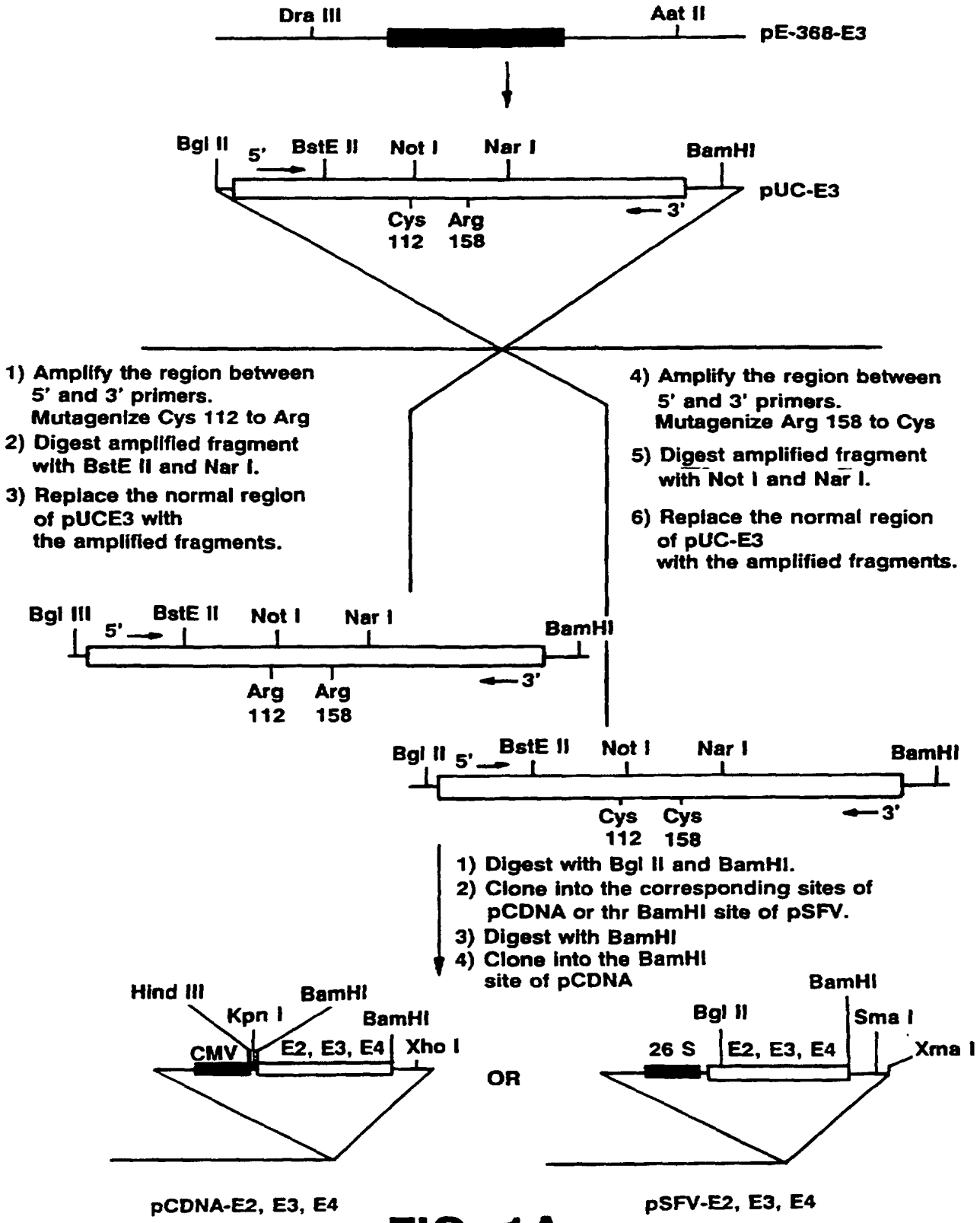

FIG. 1A

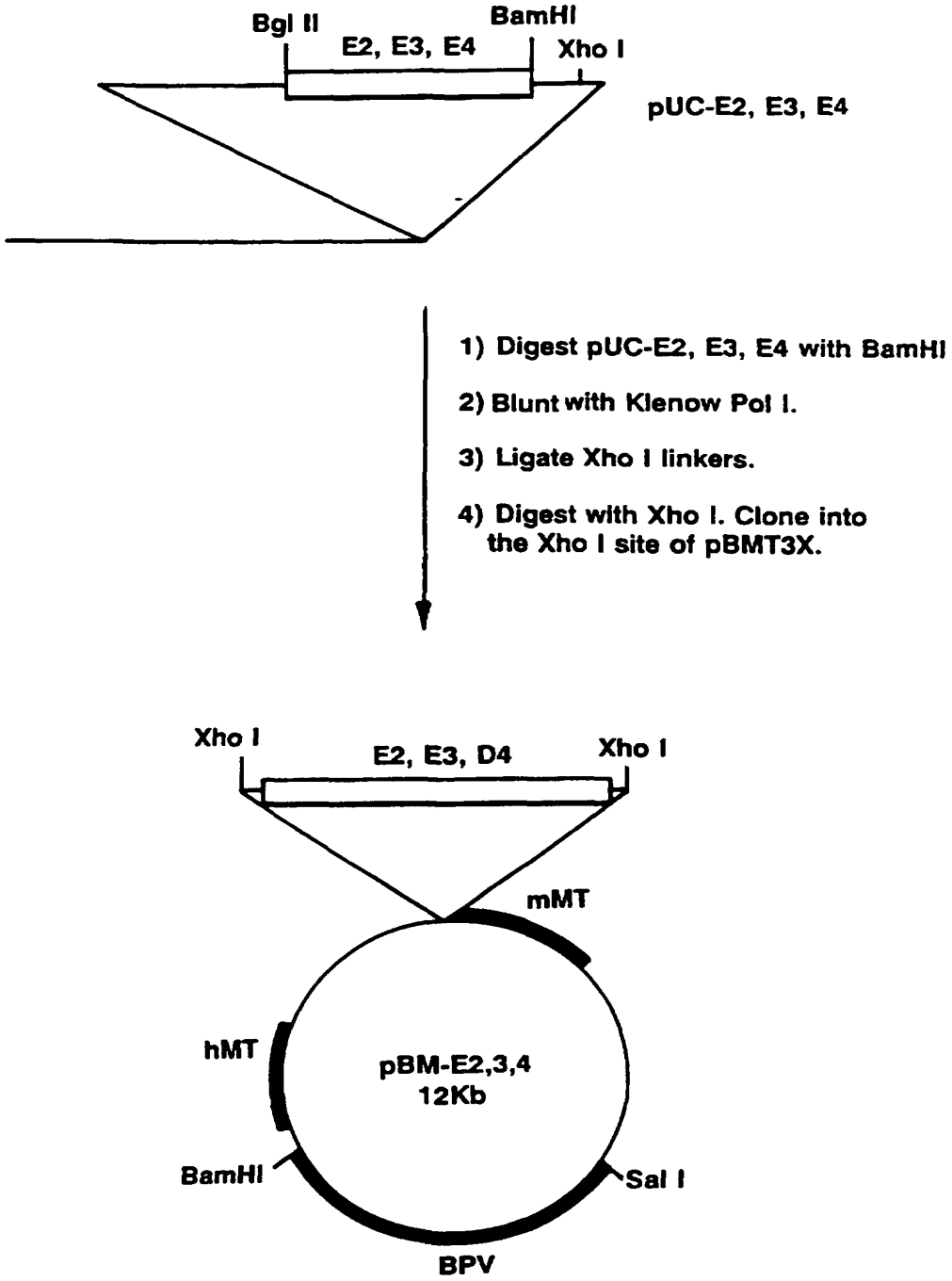
Bgl II
BamHI
E2, E3, E4
Xho I
pUC-E2, E3, E4
1) Digest pUC-E2, E3, E4 with BamHI
2) Blunt with Klenow Pol I.
3) Ligate Xho I linkers.
4) Digest with Xho I. Clone into the Xho I site of pBMT3X.
Xho I
E2, E3, D4
Xho I
mMT
hMT
pBM-E2,3,4
12Kb
BamHI
Sal I
BPV

FIG. 1B

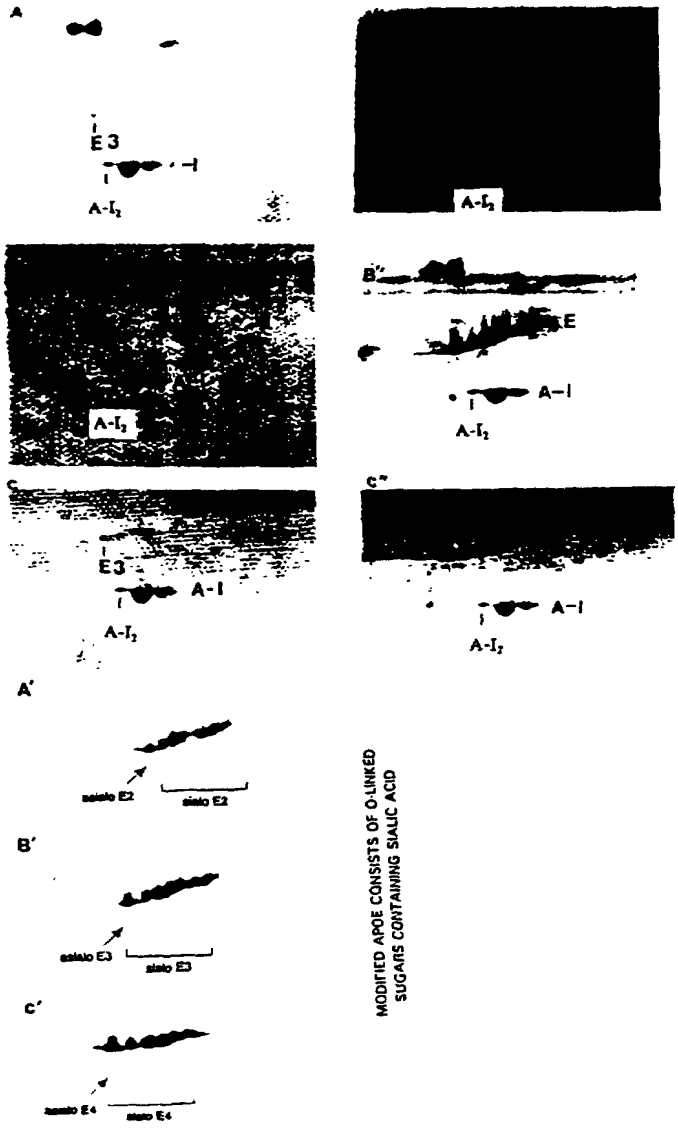
A
E3
A-I
A-I2
A-I2
B'
E
A-I
A-I2
A-I2
C
E3
A-I
A-I2
C'
A-I
A-I2
A'
asialo E2
asialo E2
B'
asialo E3
asialo E3
C'
asialo E4
asialo E4
MODIFIED APOE CONSISTS OF O-LINKED SUGARS CONTAINING SIALIC ACID

FIG. 2

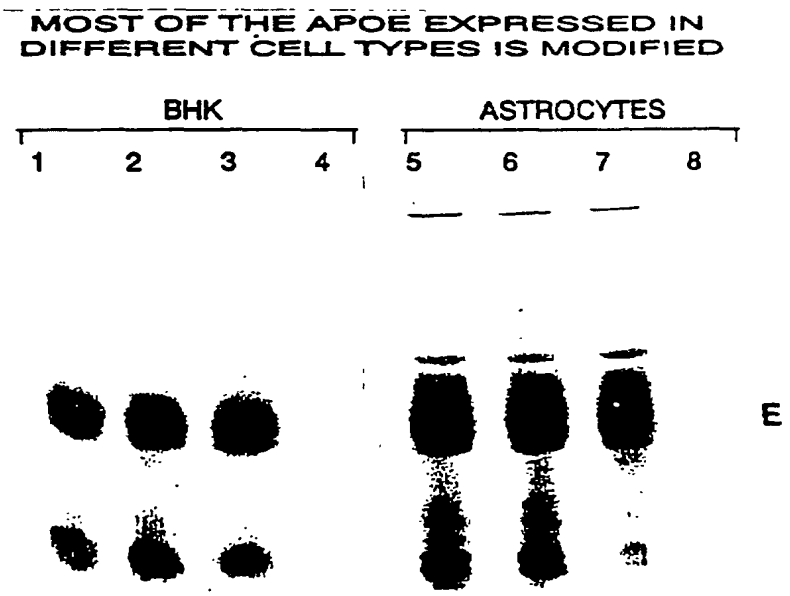

FIG. 3

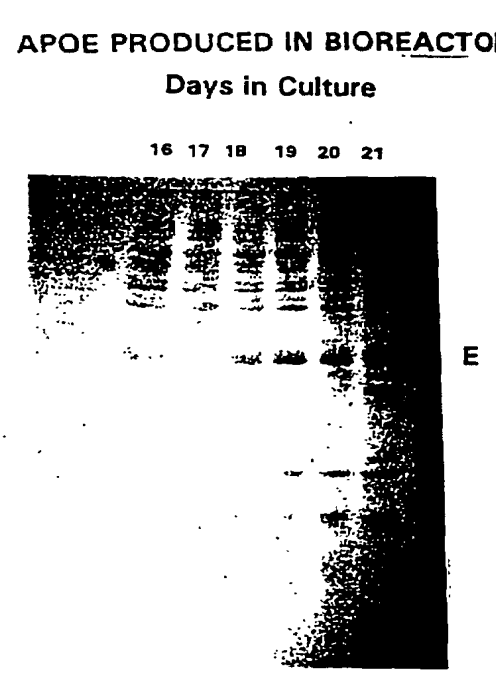

FIG. 4B

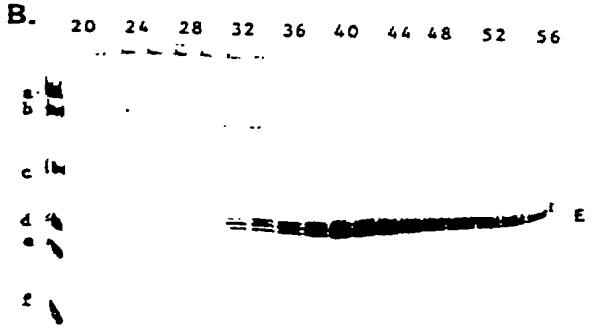

FIG. 5B

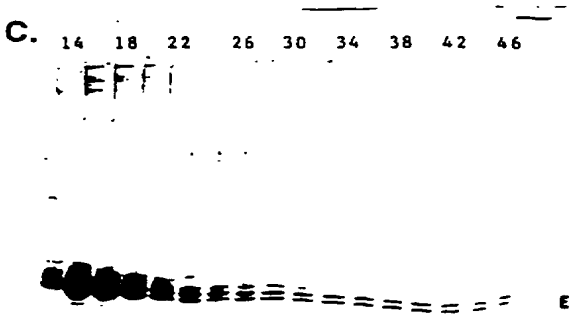

FIG. 5C

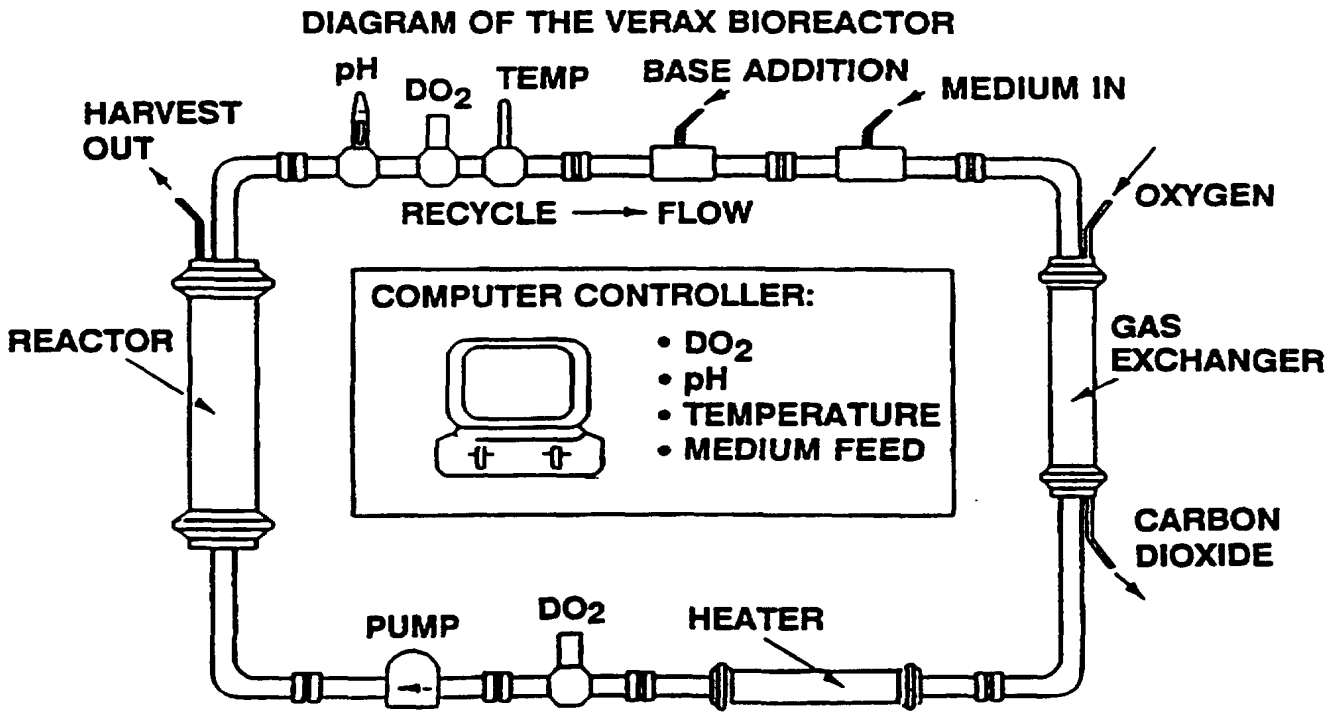
DIAGRAM OF THE VERAX BIOREACTOR
pH
DO2
TEMP
BASE ADDITION
MEDIUM IN
HARVEST OUT
OXYGEN
RECYCLE
FLOW
COMPUTER CONTROLLER:
• DO2
• pH
• TEMPERATURE
• MEDIUM FEED
REACTOR
GAS EXCHANGER
CARBON DIOXIDE
PUMP
DO2
HEATER

FIG. 4A

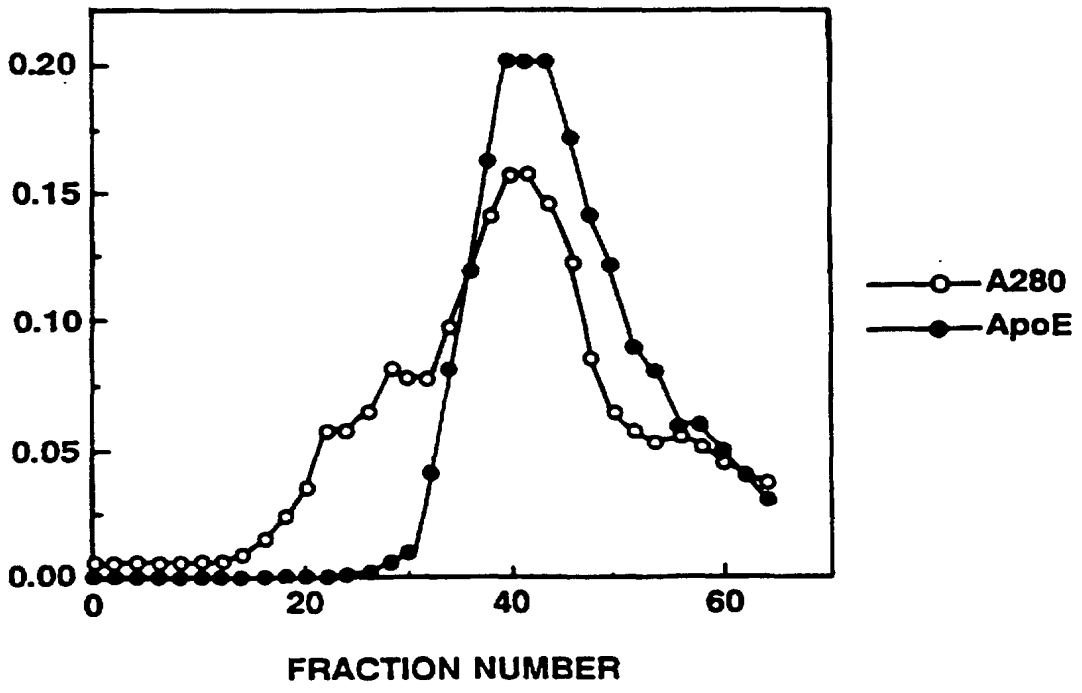
0.20
0.15
0.10
0.05
0.00
0
20
40
60
A280
ApoE
FRACTION NUMBER

FIG. 5A

THE ABILITY OF APO E ISOFORMS TO FORM COMPLEXES FOLLOWS THE ORDER: E2>E3>E4

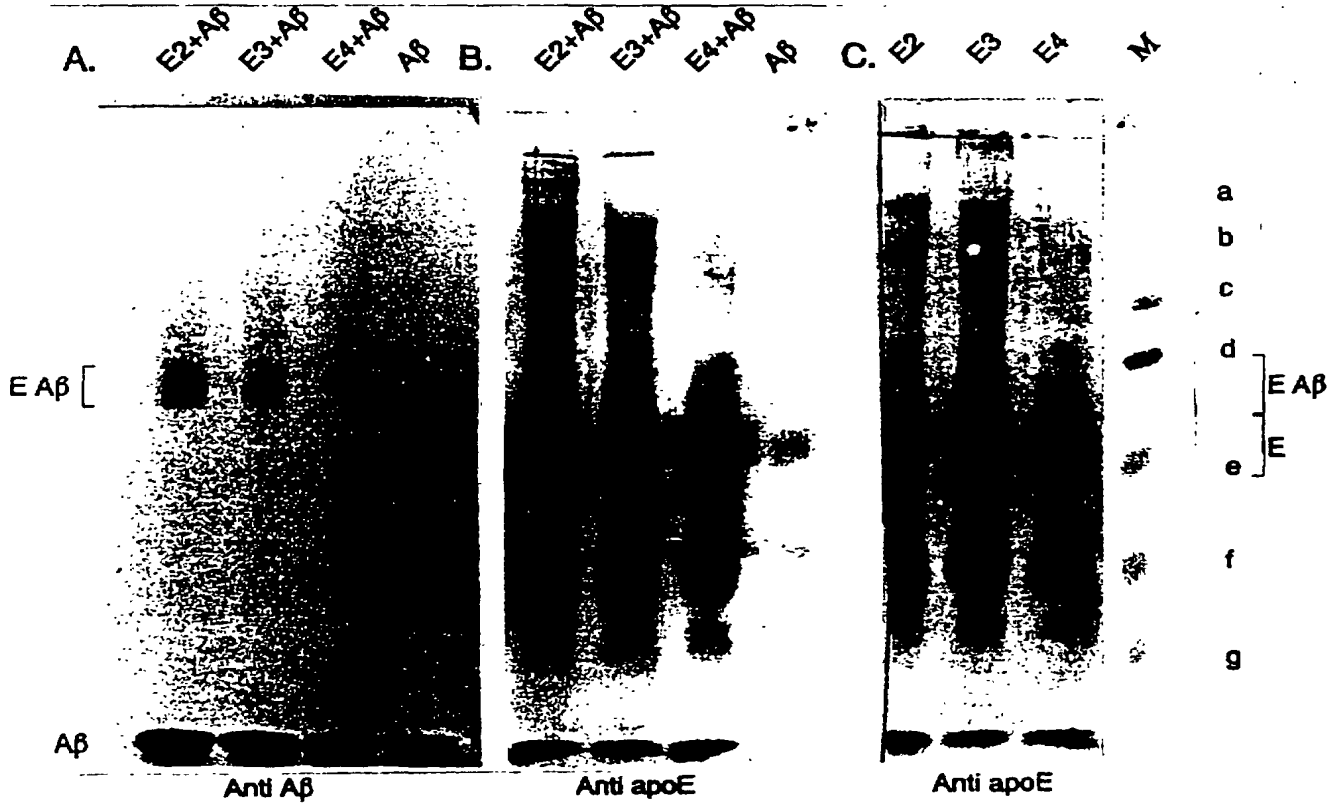

FIG. 6A FIG. 6B FIG. 6C

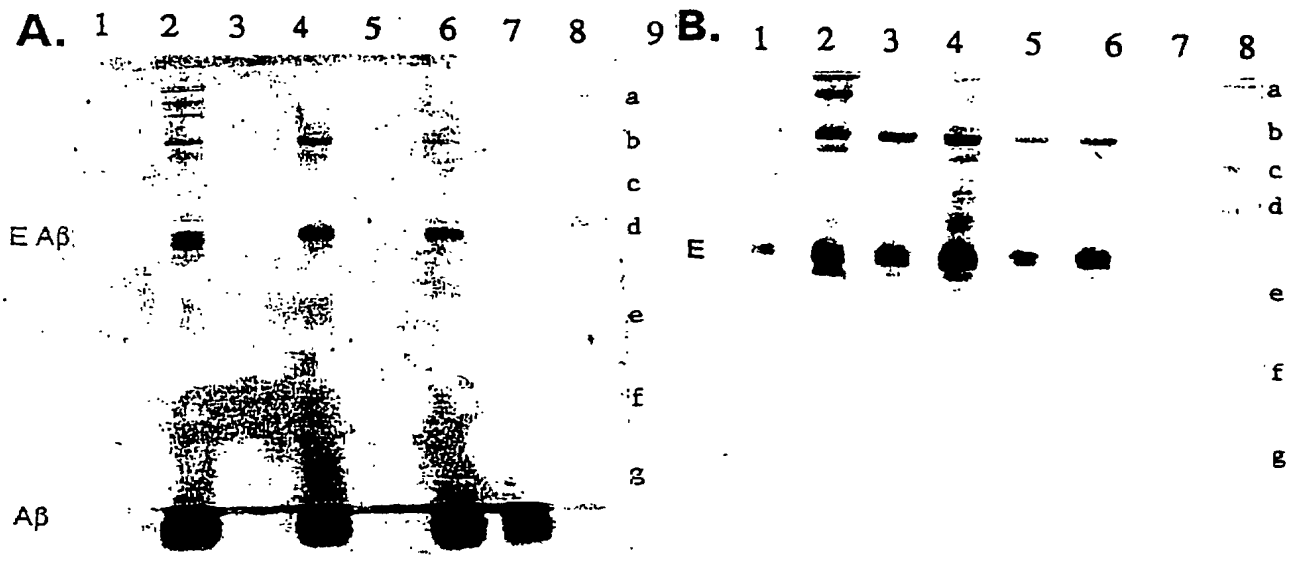

FIG. 7A FIG. 7B

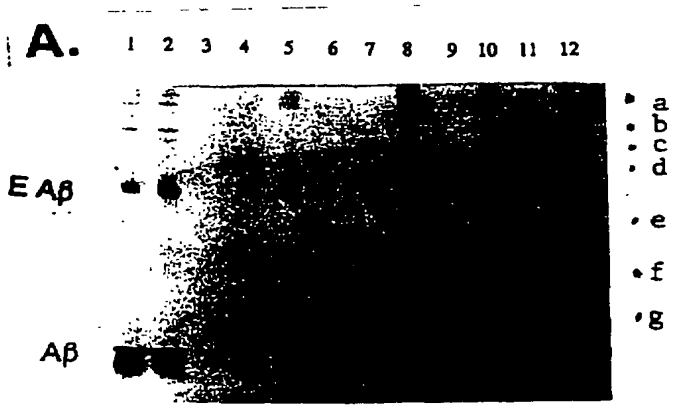

FIG. 8A

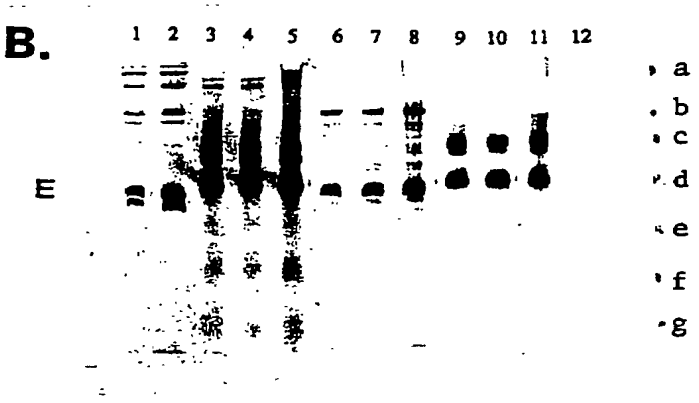

FIG. 8B

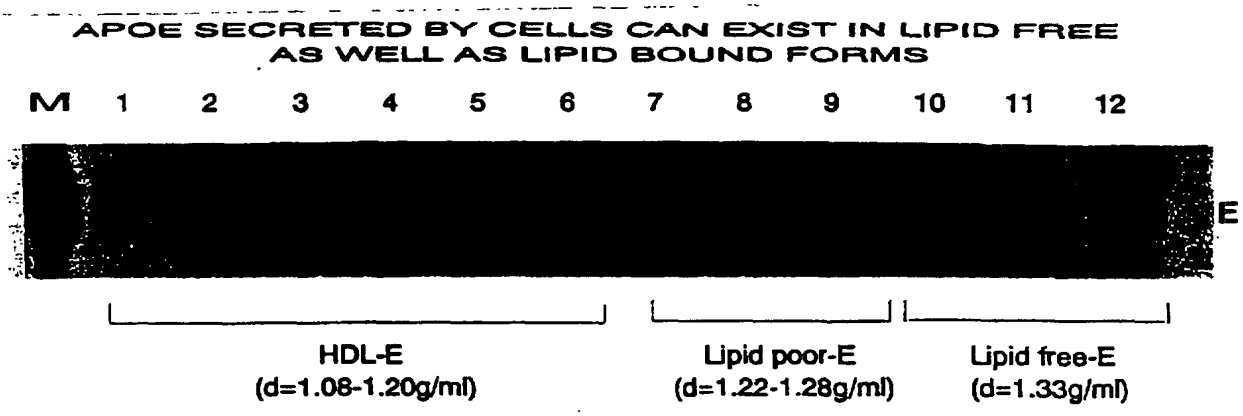

FIG. 9

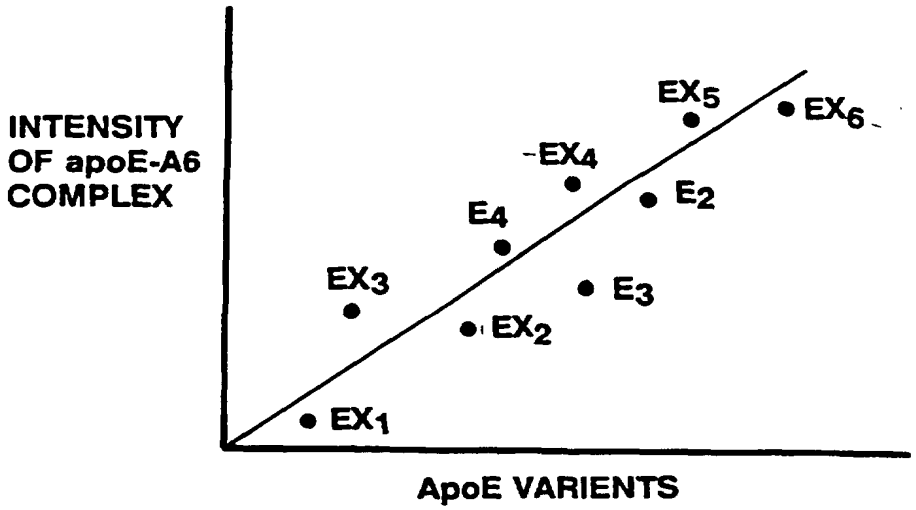

FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17629

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 1/00; C12N 5/00,15/00
US CL :530/350+; 435.240.1; 800/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350+; 435.240.1; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Chemical Abstracts, Biosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WARDELL, M.R. et al. Apolipoprotein E2-Dunedin (288 Arg->Cys): An Apolipoprotein E2 Variant with Normal Receptor-Binding Activity. Journal of Lipid Research. 1990, Vol. 31, 535-543, especially page 540.	1-7
X	MAEDA, H. et al. Molecular Cloning of a Human Apolipoprotein E Variant: E5 (Glu3 -> Lys3). Journal of Biochemistry. 1989, Vol 105, pages 491-493, esepcially page 491.	1-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
23 JANUARY 1997	13 FEB 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer DEBORAH CROUCH, PH.D.
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17629

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HERSCOVITZ, H. et al. Expression of Human Apolipoprotein E But Not That of Apolipoprotein A-1 by Mouse C127 Cells is Associated with Increased Secretion of Lipids in the Form of Vesicles and Discs. Journal of Lipid Research. 1992, Vol. 33, pages 791-803, especially pages 792 and 793.	8-10
X	PLUMP, A. S. Severe Hypercholesterolemia and Atherosclerosis in Apolipoprotein E-Deficient Mice Created by Homologous Recombinantion in ES Cells. Cell. Vol. 71, pages 343-353, especially pages 343- 345,347 and 351.	11